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Sodium retention in cirrhotic rats is associated with increased renal abundance of sodium transporter proteins

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Sodium retention in cirrhotic rats is associated with increased renal abundance of sodium transporter proteins.

Background. Liver cirrhosis with ascites is associated with a decrease in renal sodium excretion and therefore sodium retention.

Methods. In this paper, we utilize transporter-specific antibodies to address the hypothesis that dysregulation of one or more sodium transporters or channels is associated with sodium chloride (NaCl) retention in a rat model of cirrhosis induced by repeated exposure to carbon tetrachloride. Age-matched controls and cirrhotic rats were pair fed to ensure identical NaCl and water intake for 4 days prior to euthanasia for quantitative immunoblotting studies.

Results and Conclusion. The rats manifested marked extracellular fluid volume expansion with massive ascites. Plasma aldosterone levels were markedly elevated. Analysis of immunoblots revealed marked increases in the abundances of both of the major aldosterone-sensitive apical transport proteins of the renal tubule, namely the thiazide-sensitive NaCl cotransporter NCC and the epithelial sodium channel alpha subunit (α-ENaC). These results are consistent with an important role for hyperaldosteronism in the pathogenesis of sodium retention and ascites formation in cirrhosis. In addition, we observed a large decrease in cortical NHE3 abundance (proximal tubule) and a large increase in NKCC2 abundance (thick ascending limb), potentially shifting premacula densa sodium absorption from proximal tubule to loop of Henle (which powers urinary concentration and dilution).

Renal tubule sodium absorption is mediated by membrane-transport proteins that facilitate sodium movement across the plasma membranes of renal epithelial cells. In the connecting tubules and collecting ducts, sodium transport across the apical membrane is mediated by the epithelial sodium channel (ENaC) [1]. This channel is a heteromeric protein formed from alpha, beta, and gamma subunits, each of which is coded by a separate gene. In more proximal renal tubule segments, the major apical sodium transporters are the type 3 sodium hydrogen exchanger (NHE3) (proximal tubule), the type 2 sodium potassium 2 chloride cotransporter (NKCC2) (thick ascending limb of Henle), and the sodium chloride cotransporter (NCC) (distal convoluted tubule) [2]. The transport of sodium across the basolateral plasma membrane in all renal tubule segments is mediated by the sodium potassium adenosine triphosphatase (ATPase) (NaKATPase) [3].

Hepatic cirrhosis is associated with defective regulation of sodium and water. The defect in sodium and water balance appears in a sequential manner [4, 5]. At initial stages of decompensated cirrhosis, sodium and water retention by the kidney is initially isosmotic and is thought to be secondary to excessive stimulation of renal tubule salt absorption. The increase in salt absorption leads to the development of ascites. With more severe hepatic cirrhosis, free water retention can occur in excess of sodium retention leading to dilution of the extracellular fluid and the development of hyponatremia. The model of liver cirrhosis induced by repetitive CCl_4 inhalation [6] exhibits the different phases of the sodium and water retention. In our previous work, we had investigated the cirrhosis phase associated with free water retention demonstrated by a water loading test and focused on the abnormalities in protein expression attending abnormal water metabolism [7]. In the present manuscript, we investigate the changes in sodium transporter abundance along the renal tubule in the phase associated with sodium retention and ascites. We hypothesize that the increase in renal sodium reabsorption will be mediated

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by increased abundance in one of more of renal sodium transporters and channels present along the renal tubules.

METHODS

Cirrhosis model and experimental protocol

Experiments were done in adult male Wistar rats (Charles River Breeding Laboratories, Saint Aubin les Elseuf, France). Cirrhosis was induced using CCl_4 inhalation as originally described by Mc Lean, Mc Lean, and Satton [8] and modified by López-Novoa, Rengel and Hernando [9]. Phenobarbital (0.3 g/L in drinking water) was given to shorten the time required to induce cirrhosis. After 1 week of phenobarbital treatment, inhalation of CCl_4 was begun. Rats were placed in a gas chamber ($70 \times 25 \times 30$ cm). Air was bubbled (1 L/min) through a flask containing CCl_4, prior to entering the gas chamber. Animals were exposed to the CCl_4 vapor atmosphere twice a week (Monday and Friday) starting with 0.5 minutes per exposure. Afterwards, the duration of exposure was increased to 1 minute after three sessions, to 2 minutes after three more sessions, to 4 minutes after three more sessions, and then 5 minutes until the animals developed ascites. Control rats were administered the same concentration of phenobarbital in the drinking water, but were not exposed to the CCl_4 atmosphere.

Five weeks after ascites was first detected, rats receiving CCl_4 and control rats were placed in individual metabolic cages. Each rat received 14 g gelled diet per 100 g body weight per day. The gelled diet was made up of 100 mL deionized water, 59.1 g of rat synthetic food (0.083 mmol/g sodium) (Formula A04) (Panlab s.l., Barcelona, Spain), and 1.5 g of agar. The intake of sodium was therefore 0.43 mmol per 100 g per day. Rats were on this diet for 4 days before a 24-hour urine collection was initiated. After the urine collection all rats were euthanized by decapitation and the kidneys were frozen at $-80^{\circ}C$ for later processing. The protocols were performed according to the criteria of the Investigation and Ethics Committee of the Hospital Clínic. Blood was collected from the neck immediately after sacrifice and serum was separated by centrifugation.

Blood and urine analyses

Serum and urine osmolality were determined from osmometric depression of the freezing point (Osmometer 3300) (Advanced Instruments, Needham Heights, MA, USA), and sodium and potassium concentration by flame photometry (IL-943) (Instrumentation Laboratory, Lexington, MA, USA). Serum creatinine, alanine aminotransferase, total bilirubin, and total protein were determined by standard methods. The plasma concentration of aldosterone was measured with the use of a commercial kit (Coat-A-Count Aldosterone) (Diagnostic and Products Corporation, Los Angeles, CA, USA).

Polyclonal antibodies

Affinity-purified, peptide-derived polyclonal antibodies to sodium transporters and sodium channel subunits were used for immunoblotting. The antibodies used were directed to NHE3 [10], the sodium phosphate cotransporter (NaPi-2) [11], the thick ascending limb isoform, NKCC2 [12], the thiazide-sensitive cotransporter of the distal convoluted tubule, NCC [13], and the three epithelial sodium channels subunits α-, β-, and γ-ENaC [14]. The specificity of these antibodies has been demonstrated by showing unique peptide-ablatable bands on immunoblots and a unique distribution of labeling by immunocytochemistry.

Preparation of kidney tissue for immunoblotting

The rats were killed by decapitation, and kidneys were rapidly removed and chilled in cold isolation solution I [300 mmol/L of D-mannitol, 5 mmol/L of ethyleneglycol tetraacetate (EGTA), and 12 mmol/L of Tris-HCl, pH 7.4]. Control and cirrhotic rats were killed at the same time and kidneys were processed identically for both. The left kidneys were used for immunoblotting analysis, while the right kidneys were frozen immediately in dry ice for subsequent mRNA analysis (see below). The left kidneys were dissected into medullary and cortical portions and the cortices were homogenized in 15 mL of cold isolation solution I using a tissue homogenizer (PT 10-35) (Polytron Kinematica, Kriens-Luzern, Switzerland). Protein concentration was measured using the Pierce Bicinchoninic Acid Protein Assay Reagent Kit (Pierce, Rockford, IL, USA). Samples were then solubilized at $60^{\circ}C$ for 15 minutes in Laemmli sample buffer to obtain a final protein concentration of 1 μg/mL.

Aliquots of whole cortical homogenate were also processed to obtain a brush border membrane fraction using the method of Biber et al [15]. Protein concentration was measured in the brush border fractions and samples were then solubilized at $60^{\circ}C$ for 15 minutes in Laemmli sample buffer.

Electrophoresis and immunoblotting of proteins

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done using 7.5%, 10%, or 12% polyacrylamide minigels. In all cases, to confirm equality of loading among lanes, electrophoresis was initially run for the entire set of samples in a given experiment on a single 12% polyacrylamide/SDS gel which was then stained with Coomassie blue. Selected bands from these gels were analyzed by densitometry (Gel Doc 2000) (Gel Documentation System, Bio-Rad, Hercules, CA, USA) to provide quantitative assessment of loading. These loading gels established that subsequent immunoblots (loaded identically) were uniformly loaded. Proteins were transferred electrophoretically from gels

Table 1. Serum composition

	Na^+ *mmol/L*	Osmolity *mOsm/k*	Creatinine concentration *mg/dL*	Alanine aminotransferase *UI/L*	Total bilirubin concentration *mg/dL*	Total protein concentration concentration *g/L*	Aldosterone *nmol/L*
Control rats ($N = 6$)	144 ± 1	294 ± 1	0.55 ± 0.02	134 ± 32	0.15 ± 0.02	58 ± 1	0.21 ± 0.06
Cirrhotic rats ($N = 6$)	139 ± 2^a	285 ± 2^a	0.42 ± 0.05^a	281 ± 56^a	0.40 ± 0.05^a	39 ± 4^a	1.89 ± 0.73^a

Serum samples were obtained from neck immediately after sacrifice. Results are expressed as mean ± SEM.
[a] $P < 0.05$ versus control rats.

Table 2. Urinary composition

	Na^+ *mmol/L*	Na^+ excretion rate *mmol/day*	K^+ *mmol/L*	Osmolality *mOsm/k*	Water excretion rate *mL/day*
Control rats ($N = 6$)	158 ± 33	2.6 ± 0.2	203 ± 32	1580 ± 276	19.4 ± 2.7
Cirrhotic rats ($N = 6$)	39 ± 15^a	0.6 ± 0.4^a	134 ± 19	1384 ± 214	11.8 ± 1.8^a

Urine samples were collected in the final 24 hours before euthanasia. Results are expressed as mean ± SEM.
[a] $P < 0.05$ versus control rats.

to nitrocellulose membranes. After blocking with 5 g/dL nonfat dry milk, proteins were probed overnight at 4°C with the appropriate polyclonal antibody, prepared in an antibody diluent containing 150 mmol/L sodium chloride, 50 mmol/L sodium phosphate, 10 mg/dL sodium azide, 50 mg/dL Tween-20, and 1 g/dL bovine serum albumin (BSA) (pH 7.5). The secondary antibody was donkey antirabbit IgG conjugated to horseradish peroxidase (Pierce #31458) used at a concentration of 0.16 μg/mL. Sites of antibody-antigen reaction were visualized using luminol-based enhanced chemiluminescence (ECL Blotting Reagent, RPN2109) (Amersham Pharmacia, Little Chalfont, Buckinghamshire, UK) before exposure to x-ray film (Kodak #165-1579 Scientific Imaging Film) (Rochester, NY, USA). The band densities were quantitated by laser densitometry (Gel Doc 2000) (Gel Documentation System, Bio-Rad). The densitometry values were normalized to the mean of the control to facilitate comparisons.

RNA preparation

Total RNA was extracted from the right kidneys of control and cirrhotic rats as described [16] using RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA). RNA purity and concentration were assessed spectrophotometrically. RNA integrity was confirmed by inspection of ribosomal RNA bands on ethidium bromide stained agarose gels.

RNase protection assay (RPA)

The RPA was conducted as previously described for ENaC subunits [17, 18] or NCC [19]. Biotin-labeled probes for rat α-, β-, and γ-ENaC and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were constructed from plasmids to yield protected fragments of 420, 249, 190, and 140 nucleotides, respectively. Biotin-labeled probes for NCC and β-actin were 389 and 127 nucleotides in length, respectively [19]. The ribonuclease protection assay was carried out using an RPA Assay Kit (RPA III, catalogue #1414) (Ambion, Austin, TX, USA) according to the protocol recommended by the manufacturer. Approximately 25 μg of total RNA were hybridized to 1 ng of labeled probe for the ENaC assay, while 10 μg total RNA was used with 1 ng labeled probe for the NCC assay. After hybridization and digestion with RNase A and T1, the products were subjected to electrophoresis through a 5% denaturing polyacrylamide 8M urea gel buffered with Tris borate for $2\frac{1}{2}$ hours and transferred to a nylon membrane (BrightStar Plus) (Ambion). Chemiluminescence intensity was quantitated on a densitometer using Kodak software. ENaC mRNA subunit abundance was normalized to GAPDH abundance. NCC mRNA subunit abundance was normalized to β-actin.

Presentation of data and statistical analyses

Quantitative data are presented as mean ± standard error of mean (SEM). Statistical comparisons were accomplished by unpaired *t* test (when variances were the same) or by Mann-Whitney rank-sum test (when variances were significantly different between groups). *P* values less than 0.05 were considered statistically significant.

RESULTS

Characteristics of animal model

All CCl_4-treated rats included in the study developed cirrhosis, sodium retention, and ascites. There was no difference in the amount of gelled food consumed in control and cirrhotic assuring equal water, electrolyte and nutritional intake by both groups of rats. Measurement of serum and urine electrolytes and serum aldosterone are shown in Tables 1 and 2. The urinary results demonstrated a decrease in sodium excretion in cirrhotic rats, confirming the sodium retention state associated with liver cirrhosis with ascites. The decrease in sodium excretion in the cirrhotic rats occurred without an increase in serum creatinine levels, indicating that the decrease

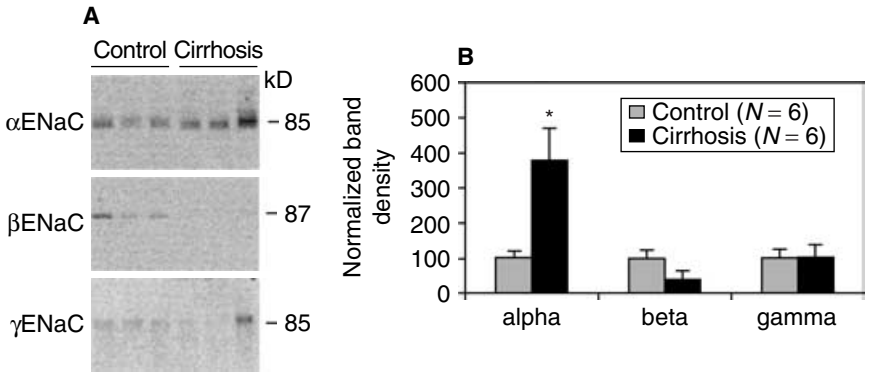

Fig. 1. Representative data showing changes in α-, β-, and γ-epithelial sodium channel (ENaC) subunits abundance in renal cortices of cirrhotic kidneys vs. controls. (*A*) Immunoblots. (*B*) Summary of densitometry data presented as percent change for cirrhosis vs. control.

in excretion was predominantly due to an increase in renal tubule sodium absorption and not to a decrease in glomerular filtration. Serum sodium concentration and osmolality were significantly reduced in cirrhotic rats, consistent with a water balance abnormality. Plasma aldosterone levels were markedly increased in cirrhotic rats from well below the k_D of the mineralocorticoid receptor ($k_D = 1.3$ nmol/L [20]) to well above it, raising the possibility that the aldosterone-regulated transporters NCC and ENaC could have contributed to the NaCl retention in this model of cirrhosis.

Post-macula densa sodium transporters and channels

ENaC levels in kidney. Semiquantitative immunoblotting was carried out to determine relative protein abundances for the three ENaC subunits in renal cortex (Fig. 1). Figure 1A shows representative immunoblots for a subset of the experiments and the bar graph in Figure 1B shows band density analysis for all rats (control, $N = 6$; cirrhosis, $N = 6$). There was a marked increase in the band density of the α subunit of ENaC in the kidneys of cirrhotic rats compared with the control rats (normalized band densities control 100 ± 20 and cirrhotic 377 ± 95) ($P < 0.05$) (Fig. 1). In addition, the band morphology was altered, with broadening of the band into the higher molecular weight region of the gel in the cirrhotic animals, suggesting increased glycosylation. In contrast, the protein abundances of the β or γ subunits of ENaC in the kidney cortex were not elevated. In fact, the level of β-ENaC was markedly decreased, much as was previously seen with dietary NaCl restriction [21] (Fig. 1). To test whether mRNA levels coding for any of the three ENaC subunits were altered in cirrhotic rats, ribonuclease protection assays were carried out. Figure 2 shows the results of these experiments. In contrast to the changes in α-ENaC protein abundance, there was no significant change in α-ENaC mRNA (band densities control 100 ± 5% and cirrhotics 186 ± 47%) ($P = 0.06$), although it might be argued that there is a "trend" for an increase. According with β- and γ-ENaC protein levels that were not modified in the kidneys of cirrhotic rats, mRNA levels for these ENaC subunits also were not altered on cirrhotic rats with ascites. The renal abundances of β-ENaC mRNA were control 100 ± 9% and cirrhotic 109 ± 15% ($P > 0.05$). The renal abundances of γ-ENaC mRNA were control 100 ± 10% and cirrhotic 61 ± 7% ($P > 0.05$).

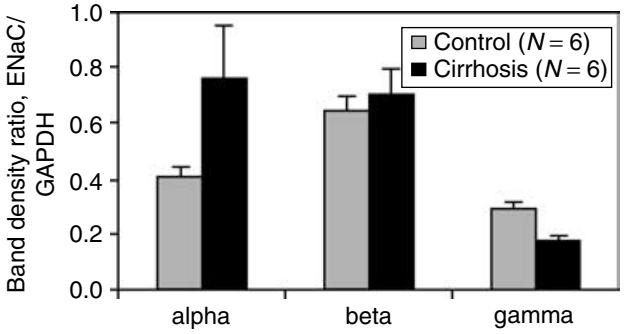

Fig. 2. Summary of densitometry data showing mRNA levels by ribonuclease protection assay for α-, β-, and γ-epithelial sodium channel (ENaC) of cirrhotic kidneys vs. controls.

NCC levels in kidney. Semiquantitative immunoblotting was carried out to determine whether NCC protein abundance was altered in kidneys from cirrhotic rats (Fig. 3). As shown in Figure 3, the band densities for NCC were increased in cirrhosis (normalized band densities control, 100 ± 13 and cirrhosis 242 ± 27) ($P < 0.05$). Because it has been previously shown that NCC protein is regulated via a posttranscriptional mechanism [19, 21], we investigated, using a ribonuclease protection assay, NCC mRNA abundance in the kidney. Figure 4 shows the results of these experiments. An example of the primary data is shown in Figure 4A and a summary of the densitometry is shown in Figure 4B. The renal abundance of NCC mRNA was decreased by about 50% (control 100 ± 9 and cirrhotic 50 ± 3) ($P < 0.05$).

Pre-macula densa sodium transporters

Although normal day-to-day regulation of sodium excretion occurs as a result of regulation of sodium transport pathways in the renal tubule segments after the macula densa feedback region (distal convoluted

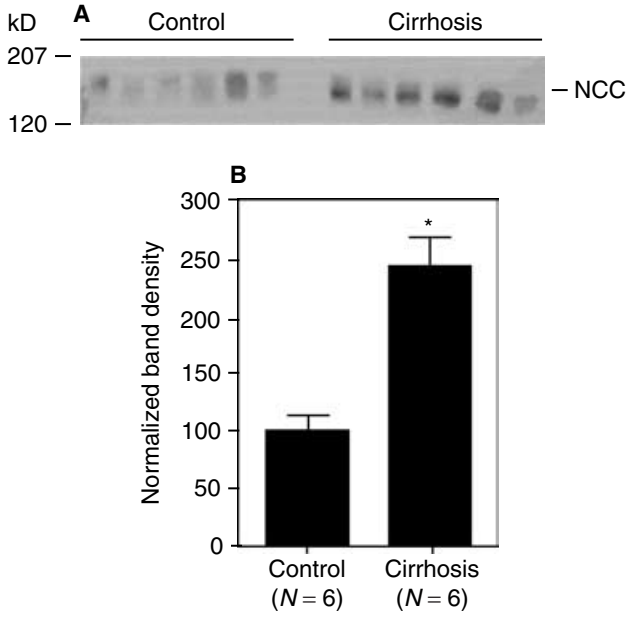

Fig. 3. Representative data showing changes of the thiazide-sensitive sodium chloride cotransporter (NCC) abundance in renal cortices of cirrhotic kidneys vs. controls. (*A*) Immunoblots. (*B*) Summary of densitometry data presented as percent change for cirrhosis vs. control.

tubule, connecting tubule, and collecting duct), alteration of sodium transport in pre-macula densa segments (proximal tubule and thick ascending limb of Henle) can conceivably play a role in dysregulation of sodium excretion if changes are large and if the tubuloglomerular feedback is impaired. Consequently, we have carried out semiquantitative immunoblotting to assess the abundances of pre-macula densa sodium transporters in cirrhotic vs. control kidneys (Fig. 5). As we have previously reported [7], CCl_4-induced cirrhosis was associated with a marked decrease in the abundance of two proximal tubule transporters, NHE3 and NaPi-2. The normalized band densities for the NHE3 signal from the renal cortex were control 100 ± 4 and cirrhotic 33 ± 22 ($P < 0.05$). The normalized band densities for the NaPi-2 signal from the renal cortex were control 100 ± 6 and cirrhotic 56 ± 3 ($P < 0.05$). In addition, there was a marked increase in the abundance of the cotransporter of the thick ascending limb, NKCC2 (normalized band densities control 100 ± 31 ans cirrhotic 483 ± 128) ($P < 0.05$).

The effects on the proximal tubule transporters NHE3 and NaPi-2 were, of course, opposite to what might be expected to explain sodium retention in cirrhosis. However, these transporters may be regulated by trafficking to and from the apical brush border membrane in addition to regulation of the total abundance of the proteins in the cell. Consequently, we prepared brush border fractions from cirrhotic vs. control kidney cortices for measurement of brush border NHE3 and NaPi-2 abundance. NHE3 protein signal from the immunoblot of the brush border fraction was not reduced in cirrhotic rats. Indeed, there was no difference between control and cirrhotic rats. The only explanation for this result is that NHE3 protein is redistributed to the apical portion of the proximal tubule cells. The densitometry values of the ratio of NHE3 band density of brush border fraction to cortex kidney homogenate were increased (control 100 ± 5 and cirrhotic 566 ± 54) ($P < 0.05$) (Fig. 6). Thus, in proximal tubule cells from cirrhotic rats, a greater fraction of the total NHE3 was present in the brush border-enriched fraction. An immunoblot of NaPi-2 from the brush border fraction showed a decrease in NaPi-2 immunoreactivity in the cirrhotic rats with ascites. The densitometry values of the ratio of NaPi-2 band density of brush border fraction to cortex kidney homogenate was control 100 ± 5 and cirrhotic 78 ± 21 ($P > 0.05$) (Fig. 6). Thus, we did not detect a redistribution of NaPi-2 to the apical brush border membrane fraction.

DISCUSSION

α-ENaC

The major new finding in this study is that the renal abundance of the α-ENaC is strongly up-regulated in CCl_4-induced cirrhosis with ascites in rats. In contrast, the β- and γ-ENaC subunit abundances were not increased; in fact, the β-ENaC subunit abundance was decreased. The α subunit has been demonstrated to be regulated by aldosterone [14, 18]. In the present study, the concentration of aldosterone in plasma was dramatically increased in cirrhotic rats compared with identically fed control rats, consistent with the view that aldosterone may have mediated the increase in α-ENaC protein in this study. Interestingly, the pattern of ENaC subunit protein and mRNA abundance changes in this model of cirrhosis differed from the pattern seen for the normal response to dietary NaCl restriction seen in rats [21]. Under NaCl restriction, α-ENaC protein and mRNA were coordinately up-regulated in whole kidney, while the abundance of the β subunit of ENaC was decreased without an accompanying change in mRNA. However, regional studies have shown that under physiologic conditions α-ENaC mRNA in the cortex does not increase substantially (e.g., during a low NaCl diet) while α-ENaC protein does increase [22]. ENaC regulation appears to be complex and may involve regulation of transcription, translation, and degradation. Weisz and Johnson [23] have suggested the possibility that noncoordinate regulation of ENaC subunits may represent another mechanism in the arsenal of physiologic responses to diverse stimuli.

There is now evidence that ENaC expression in vivo can be regulated by more than aldosterone. Two candidates that may participate in ENaC subunit regulation in the setting of cirrhosis are vasopressin and angiotensin II. Ecelbarger et al [24] have shown that α-ENaC protein

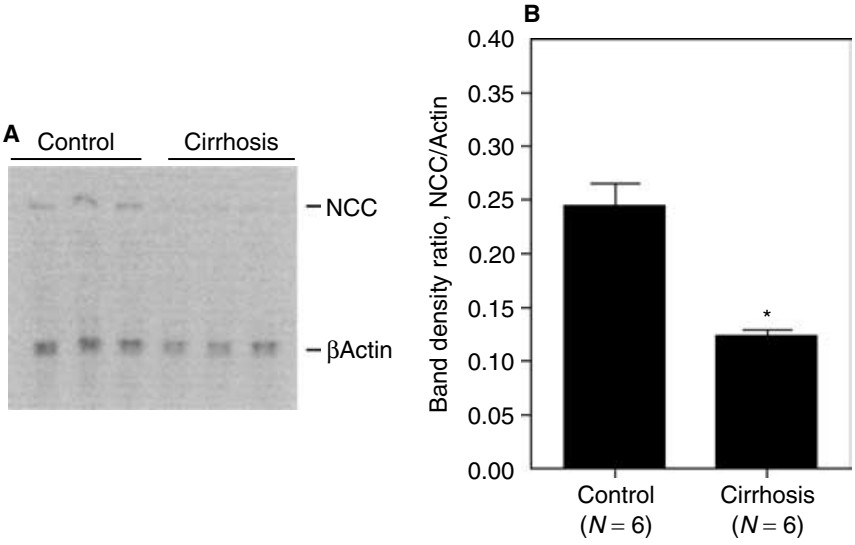

Fig. 4. Measurement of kidney thiazide-sensitive sodium chloride cotransporter (NCC) mRNA using ribonuclease protection assay. (*A*) Example of primary data. (*B*) Summary of densitometry for control vs. cirrhosis.

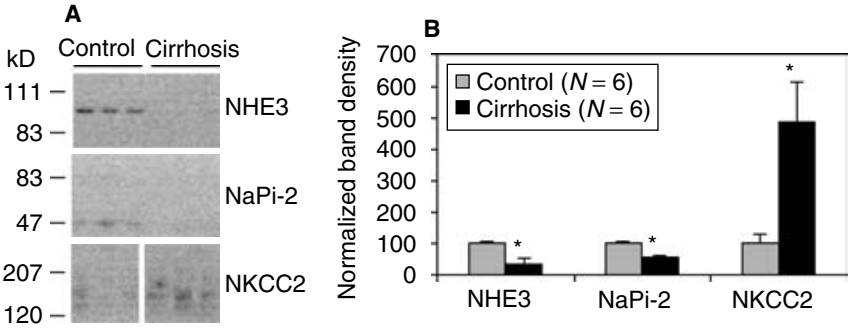

Fig. 5. Representative data for pre-macula densa sodium transporter proteins type 3 sodium hydrogen exchanger (NHE3), type 2 sodium phosphate cotransporter (NaPi-2), and type 2 sodium potassium 2 chloride cotransporter (NKCC2) abundance in renal cortices of cirrhotic kidneys vs. controls. (*A*) Immunoblots. (*B*) Summary of densitometry data presented as percent change for cirrhosis vs. control.

is increased in water-loaded rats undergoing vasopressin escape. Their results also demonstrate no increase in β-ENaC, but did show the appearance of a shorter γ-ENaC band similar to that seen in rats subjected to NaCl restriction [14]. Recently, it has also been demonstrated that angiotensin II, acting through the angiotensin type 1 (AT1) receptor, can regulate the abundance of α-ENaC in kidney [25] and we cannot rule out a role for angiotensin II in the increased expression of α-ENaC seen in this study. Although the other two subunits were not up-regulated, previous studies have suggested that the abundance of α-ENaC is rate-limiting for the formation of mature α-, β-, and γ-ENaC complexes, in renal epithelia [26].

NCC

A second transporter that was up-regulated in cirrhotic rats in these studies was the thiazide-sensitive cotransporter NCC, also a target for regulation by aldosterone [13, 27]. This result appears to be opposite to the findings that we reported for our previous study [7]. In addition it is opposite to what has recently been reported for the model of bile duct ligation [28]. Possible reasons for the differences are discussed later.

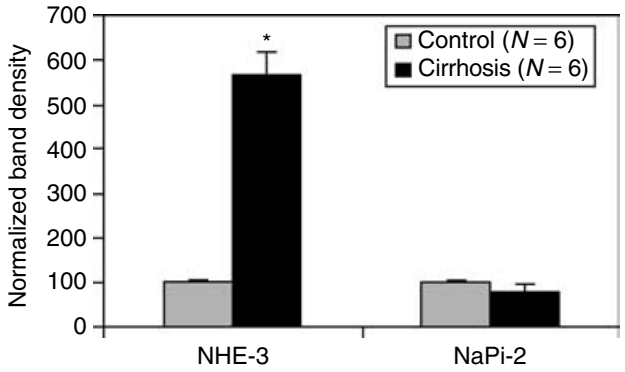

Fig. 6. Assessment of brush border vs. whole cortical type 3 sodium hydrogen exchanger (NHE3) and type 2 sodium phosphate cotransporter (NaPi-2) in control vs. cirrhotic rat kidneys. Left axis shows ratio of band densities for brush border to whole homogenate. Ratio was significantly elevated for NHE3 but not for NaPi-2.

Normally, when aldosterone-induced NaCl retention is induced under extracellular fluid volume replete conditions, the mineralocorticoid escape process is engaged, which strongly downregulates the abundance of NCC in the kidney [19]. The marked increase in NCC abundance in the present study suggests that the escape process is

impaired in this model of cirrhosis or that the escape mechanism have not fully activated. Both the primary effect of aldosterone on NCC abundance and the aldosterone escape–mediated down-regulation of NCC occur without changes in NCC mRNA [14, 21] indicating post-transcriptional mechanisms. Therefore, it is perhaps not surprising that NCC mRNA abundance was not increased in cirrhotic rats in this study. In fact, NCC mRNA abundance was markedly decreased in cirrhotic rat kidneys, indicating an underlying down-regulatory process that is evidently overcome by posttranscriptional up-regulation of NCC protein abundance.

Previous studies have shown that spironolactone, a mineralocorticoid receptor antagonist, prevents sodium retention and the development of ascites in rats with cirrhosis induced by CCl_4 and phenobarbital [4]. Recent work from Nielsen et al [27] has shown in control rats that chronic treatment with spironolactone is associated with a decrease in α-ENaC and NCC abundance in the kidney. Although not specifically investigated in this paper, it appears possible that treatment of cirrhotic rats with a mineralocorticoid antagonist may protect them from developing sodium retention and ascites by decreasing abundance of α-ENaC and NCC protein in the kidney. Further studies will be required to investigate this possibility.

NHE3 and NaPi-2

The apical component of sodium reabsorption in the proximal tubule is mediated chiefly by NHE3 [3]. Our results show significant changes in the content and distribution of NHE3 in the cortices of kidneys of rats with CCl_4-induced cirrhosis with ascites. Although there was a marked decrease in the total amount of NHE3, there was not a substantial decrease in NHE3 in membrane fractions of the apical brush border, pointing to a compensatory redistribution of NHE3 into the apical plasma membrane. The present results contrast with our previous data indicating no difference in NHE3 protein in whole kidney of cirrhotic rats. However, the present results are similar to those recently reported for the model of cirrhosis created by chronic ligation of the common bile duct [28]. These differences point to potentially important mechanisms operative under various conditions or phases of the disease (see below).

We also evaluated the response of NaPi-2, an apical sodium-phosphate cotransporter that does not play a significant role in sodium balance but, like NHE3 is believed to be regulated by trafficking to and from the apical plasma membrane. There were marked decreases of NaPi-2 abundance in both whole cortical homogenates and brush border indicating absence of redistribution of NaPi-2 into the apical plasma membrane. These results provide evidence that there are specific mechanisms involved in regulating the amount of sodium transport protein and its distribution in the proximal tubule. These mechanisms appear to be different for NHE3 and NaPi-2. The specific mechanisms involved in this synthesis and distribution are unknown. In contrast the content of NHE3 in the brush border membrane was not modified. The mechanism of these changes was not addressed in this study. In particular, they do not discriminate between the possibilities that the decrease in proximal tubule apical transporters represents a direct toxic effect of carbon tetrachloride or circulating cirrhotic toxins, or is a response to physiological mediators involved in regulation of NaCl and water balance, such as alteration in the nitric oxide or prostaglandin E_2 (PGE_2) production in the cortex. Although the proximal sodium transporters are down-regulated in the kidney of cirrhotic rats with ascites is important to point out that decreased level of a transporter does not necessarily correlate with the degree of the membrane transport.

NKCC2

Sodium chloride absorption in the thick ascending limb of the loop of Henle is mediated by NKCC2 [3]. An important observation in the present study is that the amount of NKCC2 in the kidney cortex of cirrhotic rats with ascites was markedly increased, suggesting an exaggerated sodium and chloride reabsorption in the ascending limb of the loop of Henle. This finding is in accord with previous findings of Jonassen et al [29] showing marked increases in renal NKCC2 in another rat model of cirrhosis (common bile duct ligation). Because the thick ascending limb is upstream from the site of tubuloglomerular feedback at the macula densa, sodium transport in this segment is not generally viewed as being associated with the regulation of sodium balance [30]. Rather, it plays a central role in water balance by powering the countercurrent multiplier mechanism in the medulla and the diluting mechanism in the cortex. The cirrhotic rats in the present study manifested a significant decrease in serum sodium concentration, pointing to a water balance abnormality, presumably mediated in part by increases in circulating vasopressin concentrations [31–33]. The increase in NKCC2 could conceivably be involved in the net sodium retention seen in our cirrhotic rats if the tubuloglomerular feedback mechanism is suppressed allowing a decrease in NaCl delivery to the macula densa to be sustained, without a compensatory increase in glomerular filtration rate.

The mediator of the increase in NKCC2 was not investigated in this study. However, a review of the literature reveals several possibilities: increased flow, vasopressin, nitric oxide, sympathetic nerve and rennin-angiotensin stimulation. A role for flow was inferred from a study by Ecelbarger et al [34] of the response of the thick ascending limb to administration of normal saline as a drinking solution. Since the marked suppression of NHE3 abundance in the renal proximal tubule in the present studies might

be associated with increased flow to the thick ascending limb, flow seems to be a plausible mediator. Serum vasopressin levels were not measured in the present experiment; however, circulating vasopressin levels have been a common finding in both animal models of cirrhosis and in human cirrhosis [31–33, 35]. The abundance of the NKCC2 cotransporter in the thick ascending limb of the loop of Henle is regulated by vasopressin [12]. Thus, the increase in NKCC2 abundance could have been mediated by increased vasopressin levels. Interestingly, in rats with CCl_4-induced cirrhosis with ascites and in patients with decompensated cirrhosis, the administration of V2 vasopressin antagonists induces a marked increase in urinary sodium excretion [35, 36]. A recent study by Turban et al [37] implicated nitric oxide as a factor that increases thick ascending limb NKCC2 abundance. Local nitric oxide levels tend to be high in cirrhosis in various tissues including kidney [31]. Thus, the increase in NKCC2 in the present study could have been mediated by an increased local nitric oxide levels in the region of the thick ascending limbs. Finally, it has recently been shown that the abundance of the NKCC2 cotransporter in the thick ascending limb of the loop of Henle can be regulated by sympathetic nerve and renin angiotensin stimulation [38, 39]. It is well established that in this model of cirrhosis with ascites there is a hyperstimulation of these two systems [40] that could have mediated the increase of NKCC2 in this study.

Comparison between renal abundances of sodium transporters in different phases of CCl_4-induced liver cirrhosis

We have studied two different phases of hepatic cirrhosis. In the present work we have studied rats with liver cirrhosis, sodium retention, and ascites. In our previous work, the cirrhotic rats had ascites and manifested a water excretion defect detected by a standard water loading test [7]. Consistent with the different phases of the two studies, there were a number of differences in the findings. First, rats with a water excretion defect had a decrease in the NCC level in the kidney that was not seen in the CCl_4 rats without the water excretion defect. Second, NKCC2 abundance was not altered in rats with the water excretion defect, but increased in the rats with ascites and without the water excretion defect. Third, NHE3 abundance was unchanged in CCl_4 rats with the water excretion defect but decreased in the phase without water excretion defect. However, the brush border preparation showed a redistribution of the NHE3 to the brush border. Finally, the NaPi cotransporter of the proximal tubule exhibited decreases in abundance in both phases of the disease. These differences verify that the pathophysiologic state reached in the two phases is different even though both are associated with salt and water retention. In addition, there was a difference in diet between the two studies. In the previous study, the rats were allowed to eat and drink ad libitum rather than being fed a specific measured ration as in the present study. The differences reemphasize the complexity of the mechanisms that regulate salt and water balance.

CONCLUSION

In this model of cirrhosis with ascites, the aldosterone-induced transport proteins NCC in the distal convoluted tubule and α-ENaC in the collecting duct were markedly up-regulated in the renal cortex, consistent with an important role of hyperaldosteronism in the pathogenesis of sodium retention and ascites formation in cirrhosis. It should be noted, however, that approximately 30% of patients with cirrhosis, ascites, and sodium retention have normal plasma rennin activity and aldosterone concentration. Under these circumstances, mechanisms related with reduced hepatic metabolism of some endogenous substances with sodium-retaining effects or a direct hepatorenal reflex have been suggested to account for this phenomenon [41, 42]. The current data also showed that in these rats there were reciprocal changes in the abundance of the predominant apical sodium transporters in the proximal tubule and thick ascending limb of Henle. Specifically, a large decrease in NHE3 abundance (proximal tubule) was associated with a large increase in NKCC2 abundance in the thick ascending limb, perhaps reflecting a shift in sodium absorption from proximal tubule to loop of Henle.

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